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The fission yeast meiotic regulator Mei2p undergoes nucleocytoplasmic shuttling

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Abstract ***Schizosaccharomyces pombe*** **Mei2p is an RNA-binding protein that switches the cell cycle from mitotic to meiotic. Mei2p forms a unique dot in the nucleus prior to meiosis I, aided by a non-coding RNA molecule termed meiRNA. Here we show that Mei2p intrinsically undergoes nucleocytoplasmic shuttling. Artificial acceleration of nuclear migration of Mei2p advances nuclear dot formation, but meiRNA does not appear to promote the dot formation by modulating the migration rate of Mei2p into the nucleus. Rather, this RNA is likely to facilitate the assembly of Mei2p into a dot structure and trap the protein as such in the nucleus.** **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Meiosis; meiRNA; Nuclear export; Nuclear import; Nucleocytoplasmic shuttling; RNA-binding protein

1. Introduction

An RNA-binding protein Mei2p plays a pivotal role in the regulation of meiosis in fission yeast *Schizosaccharomyces pombe* [1]. Mei2p apparently functions at two stages of meiosis. It is required for the onset of premeiotic DNA synthesis and that of first meiotic division (meiosis I) [2]. Mei2p carries three RNA recognition motifs and binds in vivo with a non-coding polyadenylated RNA molecule named meiRNA, which is essential for meiosis I [2,3]. Mei2p is cytoplasmic during the interphase, but it forms a characteristic dot in the nucleus prior to meiosis I [3,4]. meiRNA is essential for this dot formation [4].

We previously showed that integration of a nuclear localization signal (NLS) to Mei2p could bypass the requirement of meiRNA for meiosis I, restoring the nuclear dot formation in diploid cells missing meiRNA [4]. We also showed that Mei2p stayed in the cytoplasm, if it was expressed alone in cultured mammalian cells, whereas it accumulated in the nucleolus, if meiRNA was co-expressed with it. We hence speculated that meiRNA could be a cofactor to assist transport of Mei2p into the nucleus, assuming a straightforward possibility that this RNA might escort Mei2p from the cytoplasm to the nucleus [4]. Such RNA-assisted nuclear import has been demonstrated for the spliceosomal U snRNAs [5]. However, Ohno and Mattaj pointed out additional possibilities about the role for meiRNA in their review article introducing this protein–RNA cooperative system [6]. They argued that meiRNA might affect the nuclear export, if Mei2p was a shuttling protein, or might possibly be required for unloading of the cargo (Mei2p) from importin in the nucleus. Thus, we set out to address the question how meiRNA might contribute to the nuclear dot formation by Mei2p.

2. Materials and methods

2.1. Fission yeast strains, media and genetic procedures

S. pombe strains used in this study are listed in Table 1. Complete medium YE, minimal medium SD, minimal medium MM [7], and sporulation-inducing medium SPA [8] were used. General genetic procedures for *S. pombe* were according to Gutz et al. [8]. Transformation of *S. pombe* was done by a modified lithium acetate method [9].

2.2. Fluorescence microscopy

To observe localization of GFP-Mei2p in living cells, we used plasmids carrying either a *GFP-mei2* fusion gene or its variant, as previously described [4]. These fusion genes were driven by the thiamine-repressible *nmt1* promoter [10]. We also constructed *S. pombe* strains that carried a *GFP-mei2* fusion gene controlled by the *nmt1* promoter on the chromosome, in either the $sme2^{+}$ or the *sme2*Δ genetic background (JW224 and JW494, respectively). The replacement of the chromosomal *mei2* gene with the fusion gene was performed according to a standard protocol [11]. GFP-tagged Mei2p produced from the fusion gene was fully active, as judged by complementation of *mei2*Δ. Cells to be examined were cultured in liquid medium MM+N at 30°C for 16 h before microscopic observation so that the *nmt1* promoter was fully derepressed. They were harvested and subjected to fluorescence microscopy, using an Axiophot microscope (Carl Zeiss). Hoechst 33342 was used to stain nuclei in live cells.

3. Results and discussion

3.1. Inhibition of nuclear export causes accumulation of Mei2p in the nucleus

Our previous study demonstrated that Mei2p could move from the cytoplasm to the nucleus [4]. However, the possibility that Mei2p might be exported from the nucleus remained unexplored. Fission yeast exportin 1, encoded by the *crm1* gene, is known to play a key role in nuclear export in this microbe [12]. We investigated whether leptomycin B (LMB), a potent inhibitor of exportin 1 [12–16], could affect subcellular localization of GFP-tagged Mei2p. In vegetative wild-type haploid cells (JY333), Mei2p-GFP expressed from a plasmid pGFT-mei2 [4] accumulated in the cytoplasm (Fig. 1A). How-

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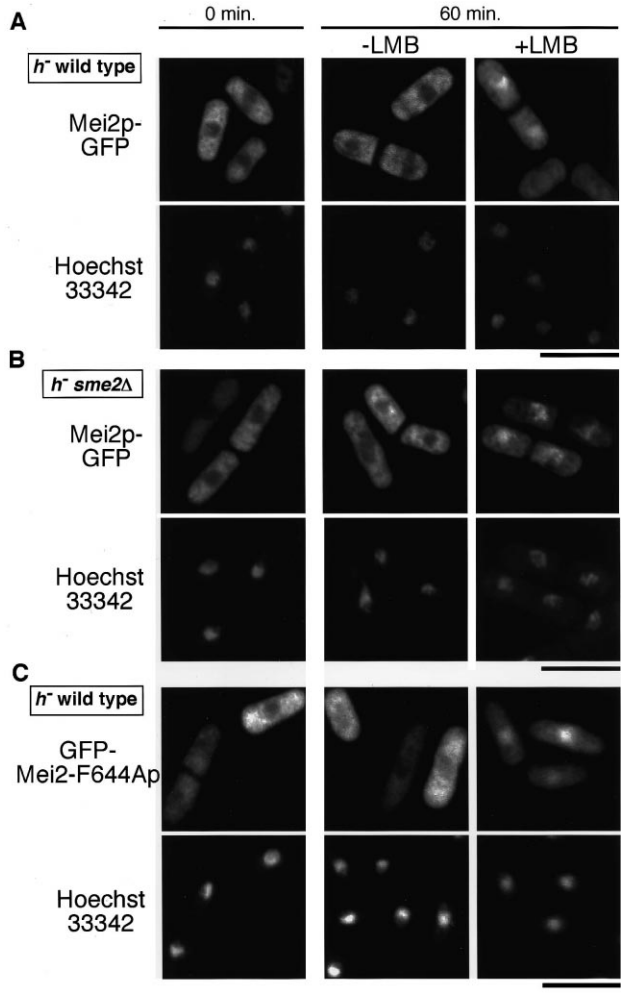

Fig. 1. Inhibition of nuclear export results in accumulation of Mei2p in the nucleus. (A) A culture of JY333 cells expressing Mei2p-GFP from a plasmid pGFT-mei2 [4] was divided into two portions, one of which received no treatment (−LMB) and the other was supplemented with LMB (100 ng/ml) (+LMB). After 60 min incubation, cells were fixed with methanol and subjected to fluorescence microscopy. Nuclei were stained with Hoechst 33342. (B) A *sme2*Δ strain JZ459 expressing Mei2-GFP from pGFT-mei2 was treated as in A. (C) JY333 expressing GFP-Mei2-F644Ap, which is defective in binding to RNA, from a plasmid pRST-mei2-F644A was treated as in A. Each bar in A, B and C: 10 μm.

ever, nuclear accumulation of Mei2p-GFP became evident when the cells were exposed to 100 ng/ml LMB (Fig. 1A). These observations suggested that Mei2p was normally subject to exportin 1-dependent nuclear export. Consistent results were obtained when nuclear export was blocked in cells of the *crm1* mutant by a shift to the semi-restrictive temperature (data not shown). We suspected that nuclear import of Mei2p-GFP might not necessarily depend upon meiRNA, because this RNA is expressed only poorly in vegetative cells [2]. To test this more critically, we expressed Mei2p-GFP from pGFT-mei2 in the *sme2*Δ strain JZ459, which does not generate meiRNA, and exposed the cells to LMB. As shown in Fig. 1B, accumulation of Mei2p-GFP was obvious in the nucleus of JZ459. Furthermore, GFP-tagged Mei2-F644Ap, a mutant form of Mei2p defective in binding to RNA [2,3], also accumulated efficiently in the nucleus of host cells (JY333) exposed to LMB (Fig. 1C). From these observations

Table 1
S. pombe strains used in this study

Strain	Genotype
JW224	*h*$^+$ *ade6-M216 leu1 kan*^{r} ≫*nmt1-GFP-mei2*
JW493	*h*$^+$ *ade6-M216 leu1 kan*^{r} ≫*nmt1-GFP-mei2 sme2::ura4*$^+$ *ura4-D18*
JY333	*h*$^-$ *ade6-M216 leu1*
JY741	*h*$^-$ *ade6-M216 leu1 ura4-D18*
JY776	*h*$^+$/*h*$^-$ *ade6-M210/ade6-M216 leu1/leu1 mei2::ura4*$^+$/*mei2::ura4*$^+$ *ura4-D18/ura4-D18*
JZ459	*h*$^-$ *ade6-M216 leu1 sme2::ura4*$^+$ *ura4-D18*

we conclude that Mei2p is a protein that can shuttle between the cytoplasm and the nucleus with no need for association to meiRNA.

3.2. Kinetics of nuclear accumulation of Mei2p

To further examine the influence of meiRNA on nuclear migration of Mei2p, we measured its nuclear accumulation semi-quantitatively, taking advantage of the blockage of nuclear export by LMB. We applied LMB to cells expressing Mei2p-GFP from the chromosomal fusion gene in either the *sme2*$^+$ or the *sme2*Δ or the *sme2*-overexpressing genetic background (JW224, JW494, and JW224 carrying pREP41-sme2, respectively). Then, the number of cells emitting GFP fluorescence more intensely from the nucleoplasm than from the cytoplasm was counted at intervals. The percentage of such cells in the population was calculated for each strain and plotted in a graph. The results shown in Fig. 2 indicate that the kinetics of nuclear accumulation of Mei2p do not differ significantly among the three strains. Apparently, overexpression of meiRNA does not enhance nuclear migration of Mei2p.

We analyzed the same samples for generation of a nuclear dot. The percentage of cells showing a clear dot in the nucleus was calculated and plotted for each strain (Fig. 2). Wild-type cells showing a nuclear dot increased proportionally as Mei2p was accumulated in the nucleus. However, the number of

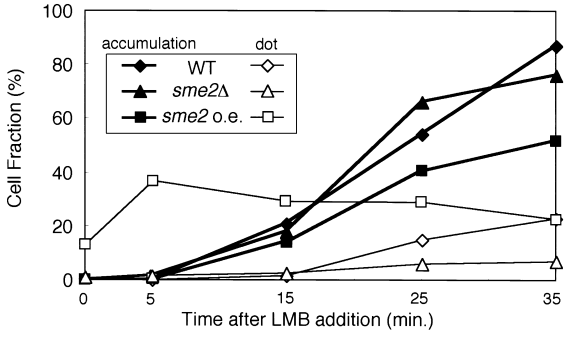

Fig. 2. Kinetics of nuclear accumulation of Mei2p in the presence and absence of meiRNA. GFP-tagged Mei2p was expressed from the *nmt1-GFP-mei2* fusion gene in JW224 (WT), JW493 (*sme2*Δ), and JW224 carrying pREP41-sme2 (*sme2* o.e.). Cells were grown in the *nmt1*-inducing medium MM+N at 30°C for 15 h. LMB (100 ng/ml) was then added to block nuclear export. At indicated intervals after the drug addition, the percentage of cells emitting GFP fluorescence from the nucleus more intensely than from the cytoplasm was measured for each strain (closed symbols with bold lines). In addition, the percentage of cells displaying a GFP-Mei2p dot in the nucleus was measured and plotted in the same panel (open symbols with thin lines). More than 100 cells were counted for each measurement. Keys for the genetic backgrounds are given in the inset.

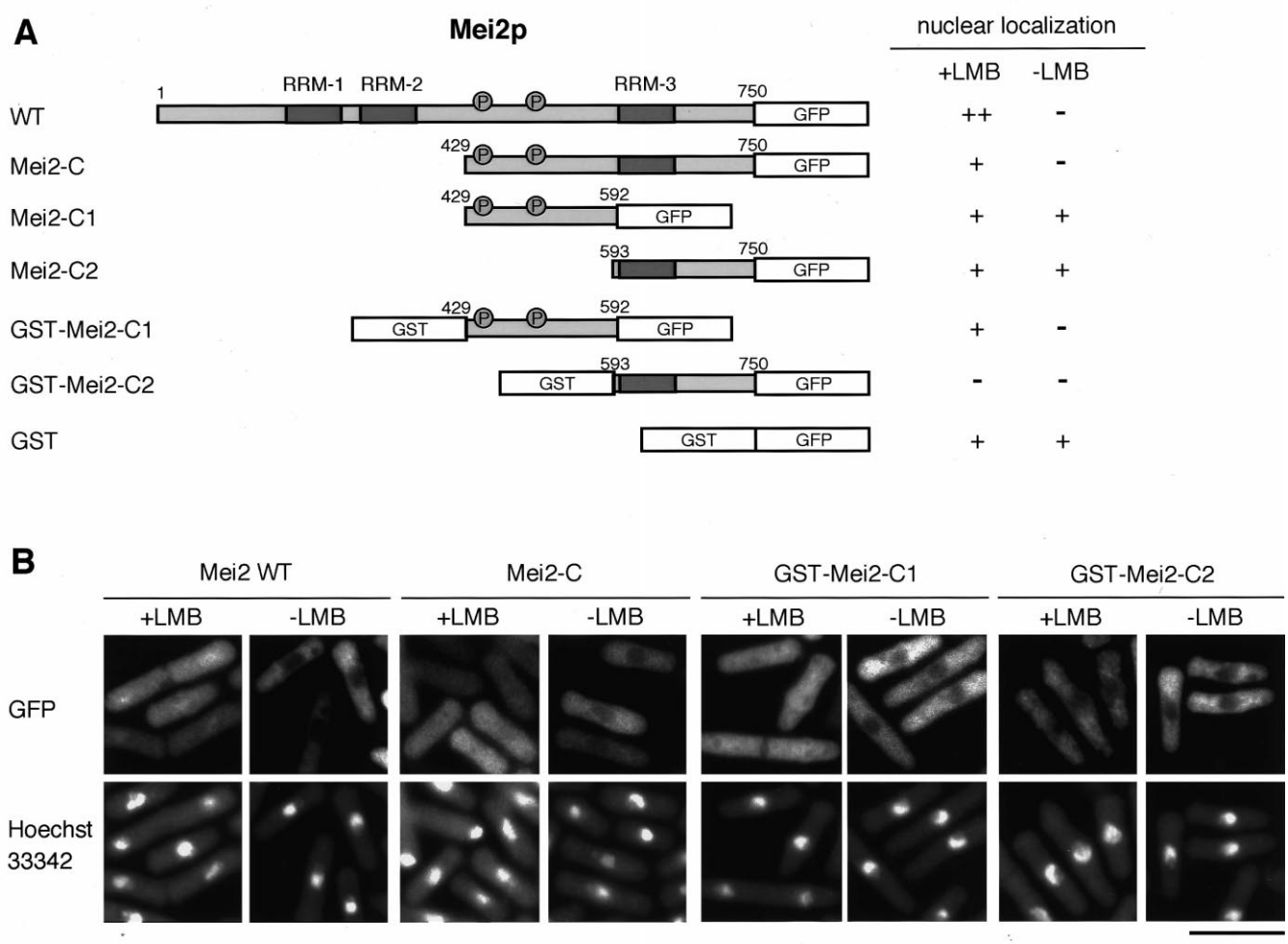

Fig. 3. Deletion analysis of Mei2p-GFP. GFP-tagged Mei2p derivatives were constructed and expressed in a diploid strain JY776. Each transformant was examined for nuclear localization of GFP fluorescence under the microscope, in the presence or absence of LMB, as described in Fig. 1. (A) Schematic illustration of the GFP-tagged Mei2p derivatives examined, with a summary of the results formulated as follows: (++) the nucleus is more fluorescent than the cytoplasm; (+) the nucleus is equally fluorescent as the cytoplasm; and (−) the nucleus is not noticeably fluorescent. P stands for the phosphorylation sites on Mei2p by Pat1 kinase. (B) Fluorescence micrographs of JY776 cells expressing either Mei2p, or Mei2-C, or GST-Mei2-C1, or GST-Mei2-C2, each tagged with GFP. Photographs were taken after 30 min incubation with or without LMB (100 ng/ml). Bar: 10 μm.

*sme2*Δ cells showing a nuclear dot increased only slightly. Although the amount of meiRNA produced in *sme2*$^+$ cells under the experimental conditions was likely to be small, these observations suggested that meiRNA had a function to promote the assembly of Mei2p within the nucleus into a dot structure. This was seen more evidently in cells overexpressing meiRNA (JW224 carrying pREP41-sme2). In these cells, the dot was visible even when Mei2p was not much accumulated in the nucleus (Fig. 2, times 0 and 5).

3.3. *Acceleration of nuclear migration of Mei2p and presence of meiRNA independently enhance formation of the Mei2p dot*

Diploid cells defective in *sme2* could form a nuclear dot and complete meiotic divisions under starved conditions, when Mei2p carrying the SV40 NLS (Mei2pNLS) was artificially expressed in them [4]. Given the above finding that meiRNA was unlikely to accelerate nuclear import of Mei2p, we set out to analyze the mechanism underlying this suppression. We expressed GFP-Mei2p and GFP-tagged Mei2pNLS (GFP-Mei2pNLS) in vegetative haploid cells, which were either wild-type, or missing meiRNA, or overproducing it. The number of cells showing a nuclear Mei2p dot was counted and their percentage in the population was calculated for each type of transformant. As summarized in Table 2, not only GFP-Mei2p but also GFP-Mei2pNLS produced a nuclear dot more readily when the expression level of meiRNA was elevated. We especially note that overexpression of meiRNA promoted formation of the Mei2p dot on top of the promo-

Table 2
Percentage of vegetative cells exhibiting a nuclear Mei2p dot

Genetic background	Expressed Mei2p derivative[a]	
	GFP-Mei2p	GFP-Mei2pNLS
Wild-type	0	4.4
*sme2*Δ	0	1.4
sme2 overexpression	14	64

[a]GFP-Mei2p was expressed from the *nmt1-GFP-mei2* fusion gene on the chromosome, whereas GFP-Mei2pNLS was expressed from the open reading frame on the vector pREP81.

tion by the SV40 NLS, while the presence of this NLS caused a nearly complete shift of Mei2p from the cytoplasm to the nucleus. These results reinforce that the promotion of the Mei2p dot formation by meiRNA is independent of the nuclear import process. In addition, they imply that meiRNA is unlikely to enhance dot formation through inhibition of nuclear export of Mei2p, although this possibility has not been rigorously excluded.

We notice that the efficiency of dot formation by GFP-Mei2pNLS in haploid *sme2*Δ cells, measured under the mitotic conditions (Table 2), is considerably lower than the efficiency for the same variant to suppress the meiosis I deficiency in *sme2*Δ cells [4]. Two lines of observation may account for this. One is that meiotic *S. pombe* cells appear to express certain RNA that can help Mei2pNLS form a nuclear dot in the absence of meiRNA [4]. This RNA is probably not effective on wild-type Mei2p, whose concentration in the nucleus is much lower. The other is that Mei2p is phosphorylated by Pat1 kinase under the mitotic conditions [1], and that the phosphorylated form of Mei2p is apparently less effective in dot formation [4]. Despite these reservations, however, the analysis done in mitotic cells demonstrates clearly that meiRNA can promote assembly of Mei2p into a dot structure in the nucleus.

3.4. Nucleocytoplasmic shuttling of Mei2p

To delimit elements responsible for the nucleocytoplasmic shuttling of Mei2p, we performed deletion analysis of GFP-tagged Mei2p. A C-terminal half of Mei2p (429–750; Mei2-C), which could perform the basic function of Mei2p [3], showed a shuttling activity, though somewhat weaker than the wild-type, as revealed by its susceptibility to LMB (Fig. 3A,B). When this segment was cut into two pieces, the central region (429–592; Mei2-C1) and the C-terminal region (593–750; Mei2-C2), connected to GFP, both appeared in the cytoplasm and the nucleus evenly in the absence of LMB, suggesting that they were small enough to diffuse freely (Fig. 3A). Similar free diffusion was observed with GST connected to GFP (Fig. 3A). To increase the size of Mei2-C1 and Mei2-C2, we connected GST to the N-terminus of each construct. The behavior of the resulting proteins suggested that the central region, which bore the two phosphorylation sites by Pat1 kinase, was likely to be responsible for the shuttling (Fig. 3A,B).

Computer search revealed no typical NLS or nuclear export signal (NES) on Mei2p. The best candidate for a NLS was RRKLR (728–732) near the C-terminus. However, consistent with the deletion analysis, alterations made in this sequence did not hamper nuclear migration of Mei2p-GFP. The best candidate for a NES was TLSASSLNPDLNLQ (555–568) in the central region. We altered its last three leucine residues to alanine, but the variant Mei2p did not cause obvious nuclear accumulation, indicating that this sequence does not play a significant role in nuclear export of Mei2p. Taken together, although the central region of Mei2p appears to play a pivotal role in nucleocytoplasmic shuttling, we have not been successful in specifying sequences responsible for it. This may mean that the NLS/NES motifs on Mei2p are rather atypical. Alternatively, the central region may interact with certain adapter proteins, which in turn interact with the nuclear import/export machinery.

The observations obtained in this study argue for that meiRNA traps Mei2p as a dot in the nucleus, rather than escorts it to the nucleus. Curiously, however, meiRNA is predominantly cytoplasmic when it is unbound to Mei2p [4]. Thus, one possible scenario is that nascent meiRNA binds to Mei2p in the nucleus and forces it to form a dot therein, otherwise meiRNA itself being exported from the nucleus. Alternatively, meiRNA may bind to Mei2p in the cytoplasm and re-enter the nucleus together with the protein. It remains to be solved where meiRNA and Mei2p meet each other and how they cooperate to assemble the dot structure. Precise mechanisms for the nuclear import and export of Mei2p also remain unanswered, except that the exportin 1-dependent pathway [17–20] apparently plays a role.

Another intriguing but difficult question remaining is the biological significance of the nucleocytoplasmic shuttling of Mei2p. One possible answer to this may be the ease of regulation. If the dot formation is limiting the rate of execution of meiosis I, it will be readily adjusted by modifying the nuclear import and/or export of Mei2p. Alternatively, the shuttling may constitute an essential part of the molecular function of Mei2p. It is presumable that Mei2p may facilitate nuclear export of certain mRNA or other RNA molecules, like hnRNP involved in mRNA export [6,19,21–24]. Further analysis is necessary also in this regard.

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